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A single natural mutation alters bacterial pathogen host-tropism

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Abstract

The capacity of microbial pathogens to alter their host-tropism leading to epidemics in distinct host-species populations is a global public and veterinary health concern. In order to investigate the molecular basis of a bacterial host-switching event in a tractable host-species, we traced the evolutionary trajectory of the common rabbit clone of *Staphylococcus aureus*. We report that it evolved through a likely human-to-rabbit host jump over 40 years ago, and that a single natural gene mutation was required and sufficient to convert a human-specific *S. aureus* strain into one which could infect rabbits at a very low inoculum. Related mutations were identified at the same locus in other rabbit strains of distinct clonal origin, consistent with convergent evolution. This first report of a single mutation that was sufficient to alter the host-tropism of a micro-organism during its evolution, highlights the capacity of some pathogens to readily expand into novel host-species populations.

Introduction

The capacity for pathogens to switch host-species leading to epidemic spread in new host populations is a major veterinary and public health concern, but our understanding of the genetic basis of host-adaptation is very limited. Many animal- and plant-associated microorganisms co-evolve with a single host species leading to complex mutualistic, commensal or pathogenic relationships, and switches between host-species must overcome innate host-specific barriers to colonization and subsequent transmission. However, successful host jumps provide an opportunity to expand into novel ecological niches, and anthropogenic events such as domestication, industrialization of agriculture, and globalization have provided increased opportunities for the transmission of bacteria between humans and animals and their subsequent dissemination¹. Recent studies have established that the host-shift and onward transmission of influenza virus requires several multi-gene mutations²⁻⁴, but the precise primary mechanisms have not been completely deciphered. The mechanisms underlying successful bacterial host-switching events are even less well understood but it is generally assumed that more complex genetic adaptations affecting multiple pathways would underlie adaptation to distantly related members of the animal kingdom.

Staphylococcus aureus is a major human pathogen that is also associated with economically important infections of livestock including dairy cows, sheep, poultry and rabbits⁵. Previously, it has been demonstrated that the major clones of *S. aureus* associated with ruminant and poultry infections evolved as the result of human to animal host jumps, leading to host-specialized endemic clones responsible for disease⁶⁻⁹. Several studies have demonstrated an association of specific mobile genetic elements (MGEs) with strains infecting particular host species suggestive of a role for MGE in host-specificity^{7,8,10-12}. However, the molecular basis for *S. aureus* host-adaptation and the underlying genetic events involved is unclear.

Results

***S. aureus* clonal complex CC121 has a multi-host tropism.** A common cause of human skin and soft tissue infections such as staphylococcal scalded skin syndrome, and life-threatening infections such as necrotizing pneumonia is the multi-locus sequence typing (MLST)-defined clone ST121¹³. Recently, the industrialization of rabbit farming in the developed world has coincided with the emergence of a highly-virulent epidemic clone of *S. aureus*, also ST121, which is associated with skin abscess and mastitis infections of rabbits in commercial rabbitries¹⁴. However, the basis for the multiple host species association is unknown. We hypothesized that the multiple host association of ST121 strains could be explained either by the existence of host-specific subtypes of ST121, or an innate capacity of ST121 strains to infect both human and rabbit hosts. In order to address this question, we carried out experimental intradermal infections of rabbits with representative ST121 isolates of rabbit (n=3) and human (n=3) clinical origin. The rabbit ST121 strains caused infections resulting in skin abscesses which are characteristic of clinical symptoms seen in natural rabbit skin infections at doses as low as 300 cfu (Fig. 1a, b and c). In remarkable contrast, human ST121 strains failed to cause clinical infections with overt symptoms even at an inoculum of 10⁵ cfu (Fig. 1a and b). These data indicate that in contrast to human ST121 isolates, rabbit ST121 strains have the capacity to cause disease in experimental infection of rabbits at very low inocula.

A human to rabbit *S. aureus* host jump occurred over 40 years ago. Given the existence of a subset of ST121 strains with the capacity to cause clinical disease in rabbits, we investigated their evolutionary history. Accordingly, we compared the whole genome sequences from a total of 23 different ST121 strains which broadly represented the breadth of spatial and temporal diversity of the ST121 lineage including 14 newly-sequenced *S. aureus* ST121 isolates obtained from humans (n=8) and rabbits (n=6) in 8 countries (from 3 continents) over a 50 year period (Supplementary Table 1), in addition to 11 diverse publicly available genome sequences for human ST121 strains¹⁵. Of note, one

of the rabbit strains (DL190) was sequenced to completion resulting in a whole genome sequence which could be used as a reference for comparison to all other ST121 sequences. We then applied a Bayesian coalescent method in the program Bayesian Evolutionary Analysis Sampling Trees (BEAST) employing a relaxed molecular clock model to reconstruct the phylogeny of the ST121 human and rabbit strains and estimate the rate of evolution of the ST121 clone¹⁶. The resulting phylogenetic tree indicates a high level of diversity of human strains represented by long branches in the ST121 phylogenetic tree in contrast to a single clade comprised of all rabbit strains which were isolated 20 years apart in 4 different countries (Fig. 2 and Supplementary Table 1). Using the calculated rate of molecular evolution for the ST121 sequences examined (1.83×10^{-6} substitutions per site per year (95% HPDs 9.84×10^{-7} - 2.26×10^{-6}), we estimated the date of the most recent common ancestor (MRCA) of the rabbit clade to be 1976 (1942-1990), and the MRCA of the rabbit clade with the most closely-related human strain F as 1909 (1804-1958). Taking into account the topology of the ST121 tree and the previously inferred human ancestry of the *S. aureus* species⁶, the most likely explanation for the existence of the ST121 rabbit clade is a single human-to-rabbit host jump which occurred more than 38 years ago leading to the emergence of a new clone associated with epidemics of rabbit farms (Fig. 2).

Diversification of the rabbit *S. aureus* ST121 core genome. Considering previous studies which have suggested an important role for MGEs in adaptation to human, ruminant and avian hosts^{7,8,10-12}, we hypothesised that the ST121 clone had adapted to infect rabbits using a similar strategy. Comparative analysis of the accessory genome of ST121 strains revealed that the majority of human strains contained MGEs which encode potent toxins involved in human disease pathogenesis such as Pantone-Valentine leukocidin (PVL) and exfoliative toxins (ET), and all but one contained a β -converting phage encoding the human-specific immune evasion cluster (IEC)¹⁷. None of the rabbit

strains contained PVL- or ET–encoding MGEs indicating their dispensability for *S. aureus* infections of rabbits (Fig. 2). Unexpectedly, the rabbit strains did not contain any MGE which are unique to rabbit *S. aureus* (Fig. 2) implying that the host tropism of the rabbit ST121 clade is not mediated by a niche-specific accessory gene pool as previously reported for *S. aureus* clones associated with poultry and ruminants⁵. Accordingly, we concluded that the capacity of ST121 to infect rabbits must have evolved through mutational events in the core genome of an ST121 progenitor of the rabbit clade. Core genome analysis revealed mutations common to all rabbit strains and absent from human strains including a total of 9 predicted pseudogenes resulting from non-sense mutations leading to premature stop codons or indels leading to frameshifts (Supplementary Table 2), and 254 non-synonymous (NS) mutations in a variety of gene loci (Supplementary Table 3). Of the genes associated with loss-of-function mutations, 4 had previously been implicated in *S. aureus* virulence including genes that would encode urease, lipase, EssB, and the global gene regulator *rot* (repressor of toxins)¹⁸⁻²⁰. In addition, a single pseudogene caused by the insertion of a prophage into the β -toxin gene (*hlb*) was identified in all human ST121 strains but was intact in the rabbit strains examined. Further, a SNP in the *dltB* gene encoding the D-alanine lipoteichoic acid and wall teichoic acid esterification protein resulted in the conversion of the stop codon into a tyrosine and extended the length of the predicted protein by a single amino acid residue. In addition, there were 2 further NS SNPs in the DltB coding sequence (Supplementary Fig. 1). Finally, an additional single 717 bp gene (*sp/E*) encoding a predicted serine protease was identified in each of the rabbits strains in the vSa β genomic island (Supplementary Table 2).

A single non-synonymous mutation is required for *S. aureus* rabbit infectivity. In order to distinguish host-adaptive mutational events among neutral or mildly deleterious mutations associated with genetic drift, mutations which were associated with encoded proteins of different size in all rabbit strains (ie resulting from frameshift, nonsense or

length extension mutations) were restored in representative rabbit strains (strains I and J) and introduced into the human ST121 strain F by allele replacement. In addition, the single gene *sp/E* found in the rabbit strains was deleted in strains I and J. The infectivity and severity of experimental infection by wild type and derived mutant strains were then compared in a rabbit skin abscess infection model. For mutations in 10 of the 12 genes examined, reversion to the human ancestral gene state in the rabbit strains and their introduction to the human ST121 strain F had no effect on infectivity or severity (Supplementary Table 2). However, reversion of the 3 identified *dltB* SNPs (*dltB^h*) in the rabbit strains J and I resulted in complete loss of infectivity, even at an infectious dose of 10⁵ cfu (equivalent to a wild type human strain; Fig. 3a). In addition, reversion of the nonsense mutation in the global virulence regulator *rot* (*rot+*) significantly reduced the severity and infectivity of the rabbit strains (Fig. 3a).

In order to examine the role of specific *dltB* mutations in rabbit infectivity, each of the 3 *dltB* mutations, in addition to the *rot* nonsense mutation, were individually introduced to the human ST121 strain F. Remarkably, introduction of the most 5' NS SNP of the *dltB* locus in the rabbit strains (T113K) to human ST121 strain F, conferred the capacity to infect the rabbit host at an inoculum of 300 cfu (Fig. 3b), causing lesions that were highly similar to that caused by the wt rabbit strain J (Fig. 3c and d). To confirm that the infective phenotype of the ST121 strain F *DltB* T113K was not due to unrelated mutations which had occurred elsewhere in the genome during the synthesis of the mutant, we restored the *dltB* gene to its original wt state in strain F and observed the expected loss of infectivity. In contrast, neither of the other 2 SNPs in the *dltB* gene (Y250H and 405Y) nor the *rot* mutation (*rot-*) increased the infectivity of the human clone F when introduced individually. Of note, the introduction of *DltB* T113K resulted in lesions in 40% of inoculated animals but bacteria were recovered from only about 20%. However, the introduction of all 3 *dltB* mutations resulted in infected lesions with 100% recovery, with an infectivity which is equivalent to the rabbit strain with a functional *rot* gene (human

ancestral state) (Fig 3 b). These data suggest that additional *dltB* mutations promote persistence of *S. aureus* ST121 in infected lesions. Importantly, a combination of the *rot* nonsense mutation with the 3 *dltB* SNPs in the human F strain resulted in a strain whose infectivity was indistinguishable from the wt rabbit strains resulting in lesions with highly similar gross and microscopic histopathology (Fig. 3b). Finally, we also verified that the rabbit strain J with a deleted *rot* gene demonstrated no further attenuation for infectivity, suggesting that loss of *rot* gene function rather than allelic diversification of the *rot* gene is responsible for the attenuation. Taken together, the data indicate that a single natural mutation of *dltB* was sufficient to confer the capacity of *S. aureus* ST121 to cause intradermal infections of rabbits, and that 2 additional NS mutations of *dltB* and loss of function of *rot* elevated the infectivity and bacterial recovery to that of wild type *S. aureus* rabbit strains.

DltB mutation-related D-Ala levels do not correlate with rabbit infectivity. DltB is an integral membrane protein encoded by the *dltABCD* operon which is likely responsible for the translocation and incorporation of D-alanine into teichoic acids and lipoteichoic acids of *S. aureus*²¹. While our understanding of the function of teichoic acids is incomplete, the addition of D-alanine (D-Ala) residues confers a positive charge which is essential for resistance to cationic peptides and for virulence^{21,22}. In view of this, we speculated that the activity of the mutated form of DltB may be enhanced leading to increased D-Ala substitution. Importantly, we confirmed that the variant DltB protein from rabbit strain J is fully functional, as a *dltB* deletion mutant was incapable of infecting the rabbit at a dose of 10⁵ cfu. Although D-alanylation of wall teichoic acids as well as resistance to the cationic peptide-mediated killing were increased in the rabbit strain compared to the human clone, this phenotype was not dependent on the *dltB* mutations (Supplementary Fig. 2a and 2b). In addition, there was no difference in D-Ala levels associated with lipoteichoic acids from strains with the different *dltb* alleles (Supplementary Fig 2c). Considering the finding that D-Ala content does not have a central impact on rabbit infectivity, we analysed if the DltB

mutations affected the bacterial cell wall composition. As shown in Supplementary Figure 3a and 3b, there were no differences in the structure or amino acid composition of the peptidoglycan obtained from the different strains. Next, considering that the DltB mutations present in the rabbit clone are predicted to have an extracellular or proximal extracellular location (Supplementary Fig. 4), we analysed if they could be involved in interacting with the innate immune system of the host or with other components of the bacterial surface. This was addressed by measuring survival in a whole blood killing assay, and by analysing the protein profile of the proteins bound to the bacterial surface. As shown in Supplementary Figure 5 and 6, although there were phenotypic differences between the human and the rabbit clones, they were not attributable to the DltB mutations. Of note is the data from the killing assays showing an increased resistance of the F *dltB*^r clone to killing compared to the wild-type F clone (Supplementary Fig. 5). However, since the mutated rabbit clone J *dltB*^h which is uninfective for rabbits did not demonstrate reduced resistance to killing compared to that observed with the J wild-type, and the survival of the J *dltB*^h clone in blood was higher to that observed with the F *dltB*^r clone, it was concluded that the ability to infect rabbits does not likely depend on the resistance observed in the killing assay. In conclusion, these data demonstrate that although we detected phenotypic differences between the human and the rabbit clones, they are not dependent on the DltB mutations. This implies that the effect of the mutated DltB on rabbit infectivity is due to alternative functions of DltB during infection. In support of this, it has been recently proposed that DltB is a member of a new superfamily of proteins, named MBOAT (for membrane-bound O-acyl transferases), that transfer organic acids, typically fatty acids, onto hydroxyl groups, performing a role in signalling²³. However, to date, O-acyl transferase enzymatic activity has not been demonstrated experimentally for DltB, and the mechanism underlying the role of the *dltB* mutations in adaptation to rabbits is unclear.

Convergent evolution of *S. aureus* at the *dltB* locus. Finally, we hypothesized that if a mutation of *dltB* was essential to change the host tropism of ST121, that similar mutations will have occurred in other *S. aureus* strains infecting rabbits and perhaps other bacterial species. Phylogenetic analysis of rabbit *S. aureus* strains based on concatenated MLST sequences indicates that the capacity to infect rabbits has occurred on numerous occasions, presumably through human-to-rabbit host jump events (Fig. 4). In order to investigate the possibility that the adaptive mutation associated with the *dltB* locus in ST121 rabbit strains is a widespread *S. aureus* rabbit host-adaptive mechanism, we sequenced the *dltB* gene of representative rabbit isolates from *S. aureus* ST1, ST8, ST9, ST45, ST96, ST133 and ST398, lineages which each include isolates of both human and rabbit clinical origin (Fig. 4a), and compared them to *dltB* sequences from diverse human and animal isolates. *DltB* was highly conserved among the great majority of human, ruminant and poultry strains of *S. aureus* with only 8 of 445 strains examined (1.8%) containing one or more NS mutations (Supplementary Fig. 1). However, 39 of 39 (100%) rabbit strains examined from diverse clonal lineages contained one or more NS SNPs in *dltB*, suggestive of convergent evolution (Fig. 4b). Of note, the majority of the mutations are also predicted to be extracellularly exposed or proximal to the outer surface of the membrane (Supplementary Fig. 4). In order to further examine the convergent host-adaptive evolution of rabbit *S. aureus* clones, we tested the role in virulence of the distinct *dltB* allele associated with the *S. aureus* ST96 rabbit clone (differing at 2 amino acid residues, Fig. 4b). Introduction of the ST96 rabbit *dltB* allele to rabbit ST121 conferred a similar level of infectivity to the ST121 rabbit *dltB* allele (10 out 15 animals infected with the ST121 allele vs 7 out 15 animals infected with the ST96 allele; $p=0.46$, Yates' chi-squared test). Taken together, these data support the conclusion that distinct *dltB* mutations occurring in different *S. aureus* strain backgrounds underpinned independent host-jump events from humans to rabbits. In contrast to the allelic diversity of *dltB* identified among rabbit strains, all rabbit strains except ST121 contained an intact *rot* gene, and the majority contained the β -converting phage, resulting in a non-functional β -

toxin, indicating that mutations affecting those determinants in ST121 are not essential for rabbit host-adaptation. Taken together, the data are consistent with the critical role for mutations of DltB in *S. aureus* rabbit host adaptation.

Finally, we identified strains of other bacterial species which had non-synonymous *dltB* mutations and predicted extended DltB proteins. These included a soil-adapted subtype of the plant bacterium *Bacillus amyloliquefaciens* which has several predicted non-synonymous mutations and an additional histidine residue at the DltB C-terminus, that were absent in the plant root-adapted strain (Supplementary Fig. 7a). In addition, analysis of publicly available *Streptococcus pneumoniae* genome sequences from a study examining resistance to vaccine and therapeutic pressures in humans, revealed additional predicted residues of DltB proteins in several unrelated strains (Supplementary Fig. 7b). Taken together, these data suggest a potential general role for allelic variants of DltB proteins in adaptation to specific environmental or host-specific niches.

Discussion.

The molecular basis of the host-adaptation of viral pathogens such as influenza and HIV has been intensively investigated. Several key mutations such as influenza PB2-627 and HIV Gag-30 have been identified as sites of critical adaptive mutations^{24,25}. However, the adaptation to mammalian hosts by both influenza and HIV appears to involve multi-gene mutations^{26,27}. For bacterial host adaptation, horizontal acquisition of a single gene regulator in the bacterial squid symbiont *Vibrio fischeri* was demonstrated to be an essential step in adaptation to its host²⁸. In addition, previous seminal work demonstrated that in a mouse model of *Listeria monocytogenes* infection, a single amino acid change in the mouse E-cadherin receptor increased *L. monocytogenes* cell internalisation *in vitro*²⁹, and conversely, 2 amino acid changes in the listerial invasion protein InlA increased *in vivo* infectivity by enhancing bacterial affinity to the murine E-cadherin³⁰. However, very high inoculum doses were still required for infectivity, suggesting that for natural *L.*

monocytogenes infections to occur additional adaptations would be required to facilitate a host shift.

To date, the fundamental biological question of how animal-bacteria partnerships are established has been difficult to dissect via established animal models of infection. In the current study, the fact that the bacterial host jump being examined involved rabbits, an experimentally-tractable host-species, facilitated for the first time, a dissection of the evolutionary genetic trajectory of a natural host-species switch by a bacterial pathogen. Using this approach, we traced the evolutionary genetic events which led to the emergence of a major animal clone of *S. aureus* responsible for disease epidemics of farmed rabbits on a global scale. In contrast to all characterized human and animal clones of *S. aureus*, adaptation to the rabbit host did not involve acquisition of MGE from a host-specific accessory gene-pool. Remarkably, we report that a single nucleotide mutation, which occurred naturally, was sufficient to allow a radical change in bacterial host-tropism. The mutation, affecting the *dltB* gene was sufficient and required to convert a human *S. aureus* strain, which was incapable of causing infections in rabbits, to one that had the capacity to cause epidemics in farmed rabbit populations. An additional 2 mutations of the same locus enhanced infectivity and bacterial proliferation within rabbit skin lesions. In spite of apparently numerous host jump events into rabbits, ST121 is by far the dominant clone in the countries sampled. As such, we suggest that in addition to the initial adaptations required for survival in the new host species, additional mutations likely contribute to the transmissibility and successful spread of the infectious clone.

In summary, our results reporting a single natural mutation associated with a bacterial host switch event represent a paradigm shift in understanding of the minimal adaptations required for a bacterium to overcome species-barriers and establish in new host populations. The discovery has important public and veterinary health implications which will require a re-examination of the future threat posed by bacterial pathogen host switching events.

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Author Contributions. J.R.F. and J.R.P. conceived and designed the study; D.V., M.C. and L.S. generated and characterized the different mutant strains; P.R.M, M.J.W. and C.M.G performed the genomic studies; B.M.G and S.J.F. measured D-Ala content; A.T. provided human strains; J.R.P, J.R.F and S.J.F supervised the research; J.R.F. and J.R.P. wrote the manuscript; J.R.F. and J.R.P. obtained funding.

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Figure legends

Figure 1. Rabbit but not human ST121 strains can infect rabbits. (a) Percentage of rabbits infected with the rabbit or human ST121 clones (day 7 after inoculation). Rabbits (n = 24 per strain) were inoculated intradermally with either *S. aureus* ST121 rabbit strains I, J or DL190 (300 cfu) or human strains G, F or A (10⁵ cfu), as described in Methods. Yates' chi-squared test was used to compute P values for between-group comparisons; differences that are statistically significant are indicated by an asterisk (P < 0.0001); all other comparisons were non significant. **(b)** Representative rabbit skin lesions (day 7 post-inoculation). Images of gross and microscopic histopathology are presented for representative animals as follows: panel 1, gross skin pathology. Bar = 1cm; panel 2, transversal section of skin lesions. Lesions resulting from infection with rabbit strains were characterized by dermal abscesses (da) up to 2 cm in diameter, with epidermal ulcers (u) in the most severe cases. Bar = 1cm; panel 3, H&E-stained sections of the skin inoculated with human or rabbit ST121 clones, respectively. Bar = 0.5 cm. Microscopically, areas of purulent material surrounded by fibrosis infiltrated by inflammatory cells were observed. **(c)** Experimental skin lesions (day 10 post-inoculation) were indistinguishable from natural lesions. Panel 1, gross skin pathology; panel 2, transversal section of skin lesions. panel 3, H&E-stained sections of the skin. Bar = 0.5 cm.

Figure 2. Evolutionary history of the ST121 clonal complex suggests a human-to-rabbit host jump leading to the emergence of an epidemic rabbit-specific clone.

Bayesian phylogenetic reconstruction of the CC121 lineage based on core genome alignment with branches color-coded according to host species association (blue, human; red, rabbit). The presence or absence of mobile genetic elements (MGE) in the accessory genome is denoted by filled or empty squares, respectively. MGE identified include Staphylococcal Pathogenicity Islands (SaPI), phages (ϕ), or plasmids (p) containing genes: *lukF/S*, Pantone-Valentine leukocidin; *eta*, exfoliative toxin A; *etb*, exfoliative toxin B; *seb*, staphylococcal enterotoxin B; IEC, immune evasion cluster; *SCCmec*, staphylococcal

cassette chromosome *mec*, and plasmids encoding resistance to bleomycin, kanamycin, quaternary ammonium compounds and trimethoprim (pR1) or teicoplanin (pR2), respectively. Branch lengths are scaled according to the time-scale indicated on the x-axis.

Figure 3. A single mutation is sufficient to confer rabbit infectivity to a human *S. aureus* strain. Percentage of rabbits infected with the different rabbit (a) or human (b) ST121 mutants (day 7 after inoculation; n = 20 per strain in panel a, n = 30 per strain in panel b). Panel (b) also shows the percentage of animals that developed skin lesions by the inoculated bacteria. Yates' chi-squared test was used to compute P values for between-group comparisons; differences that are statistically significant are indicated by an asterisk ($P < 0.05$); all other comparisons were non significant. (c) The evolution of the skin lesions produced by the rabbit strain J and by the human clone carrying the DltB T113K mutation was indistinguishable. Rabbits were inoculated intradermally with 300 cfu of either the rabbit wt or the human mutant clones, as described in Methods. A representative animal is shown. The earliest gross changes were observed 24-48h post-infection (p.i.), consisting in a slight increase in size and erythema, but evolving to form skin abscesses up to 2 cm in diameter (7 day p.i.). Bar = 1,5 cm. (d) Transversal section of the skin lesions. Lesions produced by the rabbit and human mutant clones were characterized by dermal abscesses (da) up to 2 cm in diameter. Bottom: H&E-stained sections of the skins inoculated with the rabbit or human mutant ST121 clones. Bar = 0.5 cm. Microscopically, areas of purulent material surrounded by fibrosis infiltrated by inflammatory cells were observed.

Figure 4. Rabbit *S. aureus* clones have evolved on numerous occasions and are associated with NS mutations of *dltB*. (a) Bayesian phylogeny of *S. aureus* species reconstructed using nucleotide sequence of five non-recombinant MLST loci from 108 STs representing the breadth of species diversity. Clonal complexes/sequence types with known rabbit host association are indicated with red circles, and scale bar represents 5

nucleotides. **(b)** Non-synonymous polymorphisms identified in the *dltB* locus from rabbit associated clonal complexes in comparison with *dltB* from human-associated *S. aureus*. In bold are shown the residues affected in multiple clones.

METHODS

Bacterial strains and growth conditions. Bacterial strains used are listed in Tables S1 and S4. *S. aureus* strains were grown at 37°C on TSA agar medium, supplemented with antibiotics as appropriate. Broth cultures were grown at 37°C in TSB broth with shaking (240 r.p.m.). LB agar and LB medium were used for *E. coli* strains, supplemented with antibiotics as appropriate. Procedures for transduction and transformation in *S. aureus* were performed as described previously^{31,32}.

DNA methods. General DNA manipulations were performed by standard procedures. To introduce specific mutations into *S. aureus* strains, we used plasmid pMAD³³ for allelic exchange as previously described³¹. Plasmid constructs (listed in Table S5) were prepared by cloning PCR products obtained with oligonucleotide primers as listed in Table S6, and all clones were sequenced by the Institute Core Sequencing Lab.

Whole genome sequencing and alignment. Isolates were sequenced using a combination of single and paired end technologies on the Roche 454 platform and assembled using the Roche GS De Novo Assembler v2.6, or alternatively using the Illumina HiSeq with reads assembled using Velvet v1.2.08. Contigs were aligned using the progressiveMauve algorithm, filtered for locally colinear blocks over 1000 nucleotides in length, and gap positions were removed³⁴. The alignment was assessed for recombination using BRATNextGen³⁵, with predicted recombinant regions excluded from further phylogenetic analyses. Genes specific to rabbit ST121 isolates were identified by comparing the sequenced rabbit isolate genome sequences to the human ST121 sequences using the print_novel_contigs function of cortex_var v1.0.5.20³⁶.

Bayesian evolutionary analysis. Based on the core genome Mauve alignment (6208 variable sites), the phylogeny of ST121 was reconstructed using BEAST v1.6.1¹⁶ under the GTR model of nucleotide substitution with a gamma correction for rate heterogeneity and a skyline coalescent tree prior. Simultaneously we used an uncorrelated lognormal distribution to model the rate of evolution and constrained the tips of the phylogeny to their dates of isolation to calibrate the rate³⁷. Posterior probabilities were estimated from 3 independent Markov chain Monte Carlo (MCMC) samples from 3 independent analyses each run for 1×10^8 iterations, with sampling at every 1000 generations and 10% discarded as burnin.

Identification of pseudogenes and non-synonymous mutations. Single end 454 sequence reads were aligned to the ED133 genome (accession number NC_017337.1), and an annotated draft version of the DL190 genome using the BWA long read aligner³⁸. Point mutation and insertion/deletion variants were called at sites with average mapping and base quality scores greater than 30. Mutations predicted to result in pseudogenes were further inspected manually using the Integrative Genomics Viewer³⁹. Variation in gene content between strains was examined using the GeneFamily method of the Pangenome Analysis Pipeline with default parameters⁴⁰. Presence or absence of virulence factors was confirmed through manual inspection of the output of BLAST searches.

Rabbit skin infection model. Two-month old albino hybrid rabbits of either sex were used for the skin infection model. Rabbits were sedated with a combination of ketamine and xylazine and were shaved on their back and inoculated by intradermal injection (usually with 300 cfu of *S. aureus* in 100 μ l of PBS). Each rabbit was inoculated in duplicate with 2 strains (generally wild type and mutant), and each strain was tested in groups of at least 20 rabbits. After 7 d, rabbits were euthanized by intravenous injection of barbiturate (Dolethal, V  toquinol SA). In each experiment, an additional group of animals was inoculated with vehicle (PBS) and served as a negative control. In addition, to

exclude the possibility of contamination, bacteria recovered at the end of the experimental period were analysed by sequencing of strain-specific gene alleles. The experimental protocol was approved by the ethical committee of the Universidad CEU Cardenal Herrera and by the Conselleria d'Agricultura, Pesca i Alimentació, Generalitat Valenciana (permit number 2011/010).

Macroscopical and histological examination of lesion tissues. Rabbits were examined daily and development of infection was followed visually and by palpation of lesion tissue. Length and width values were measured to calculate the area of lesions. Addition of lesion size, skins were also examined to evaluate presence or absence of epidermal necrosis. After post-mortem examination, skin abscesses were routinely processed for histological examination and stained with haematoxylin and eosin and by Gram's method. Previously fixation, swab samples were taken from lesion and cultivated on blood-agar (BioMérieux, Marcy l'Etoile, France) to confirmed presence of *S. aureus*.

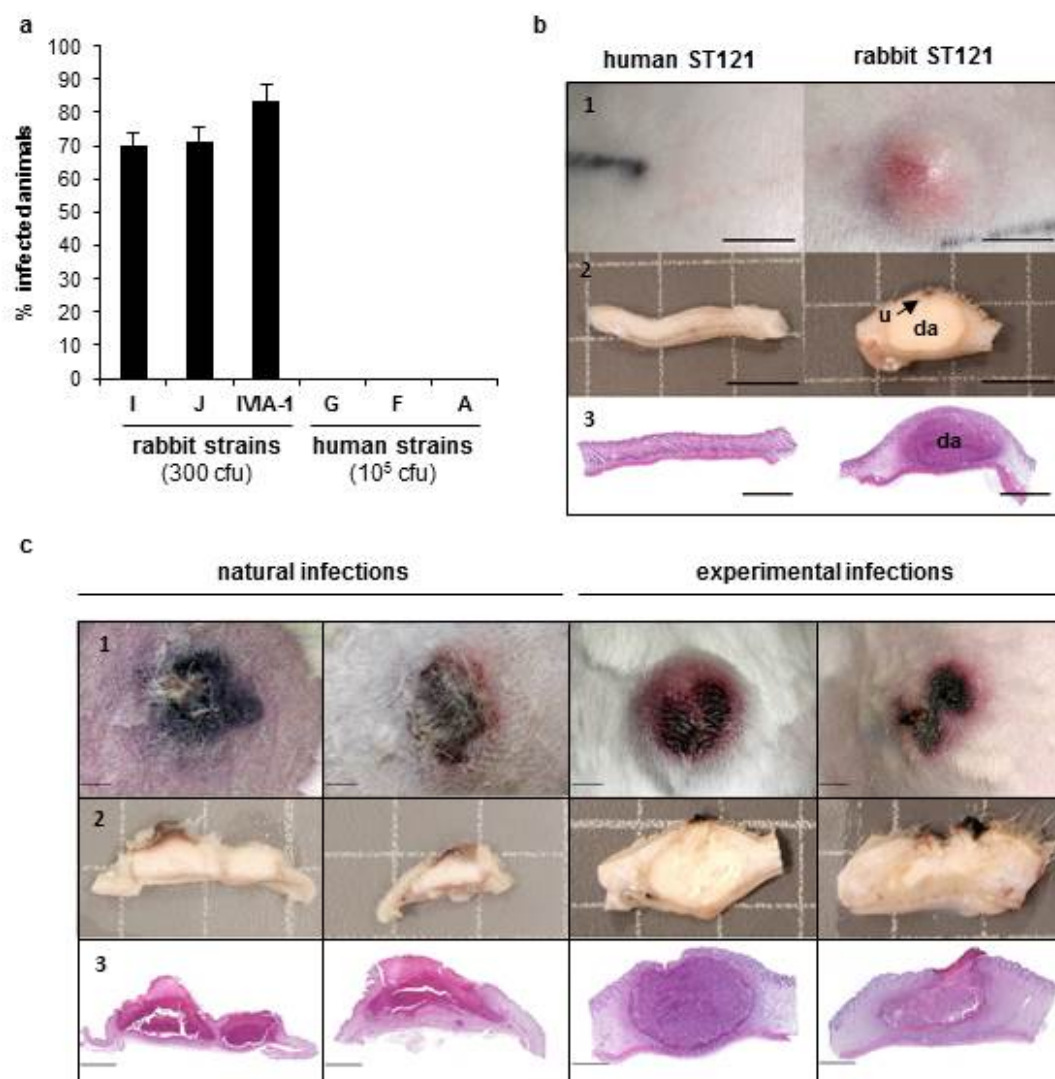
Cell wall and D-alanine analyses. Cell walls were isolated, wall teichoic acids and lipoteichoic acids were purified and D-alanine content measured as previously described^{21,41}. The peptidoglycan composition and structure in the different strains was analysed by Cecolabs (Tuebingen, Germany), as described before⁴².

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Fig. 1



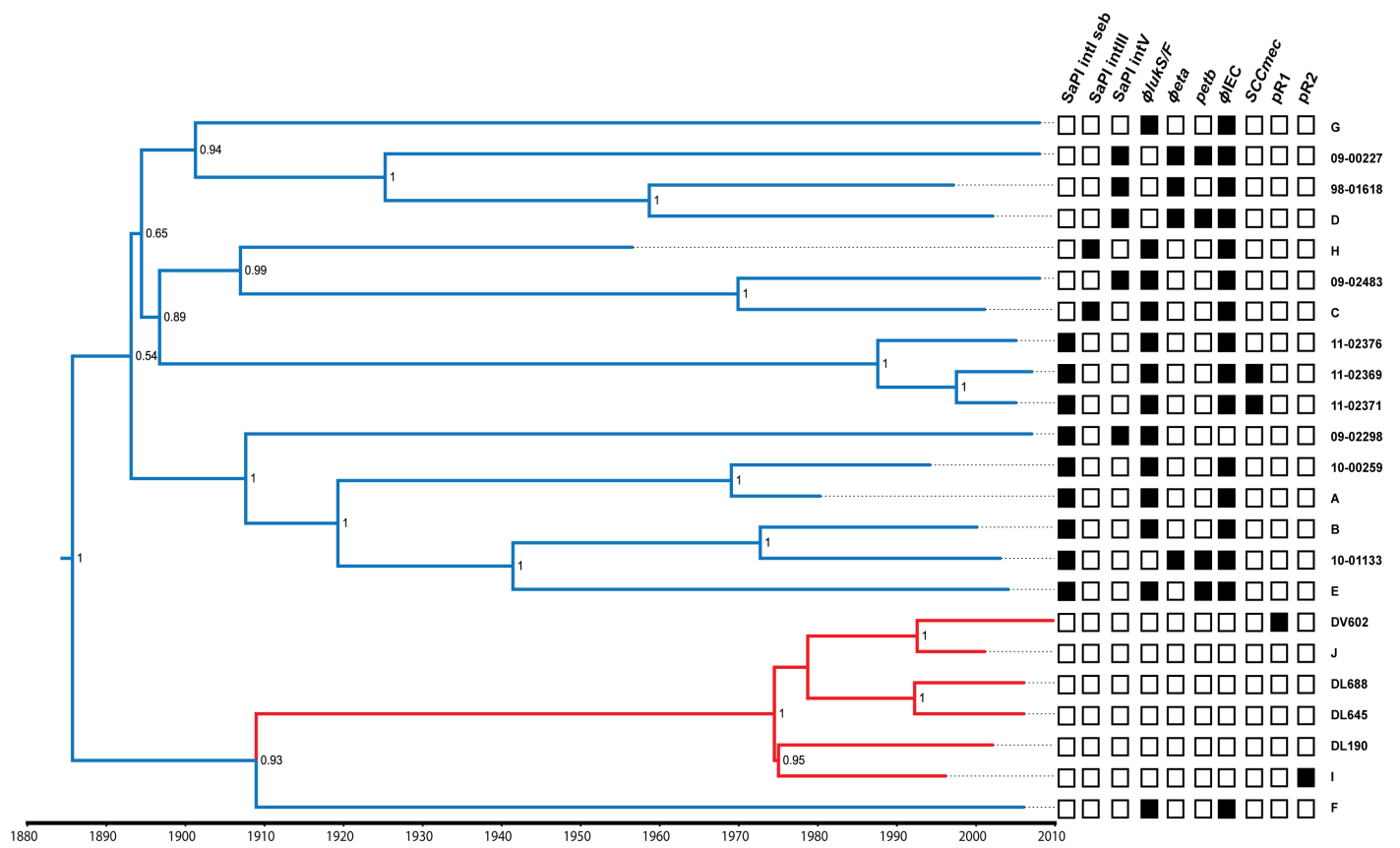
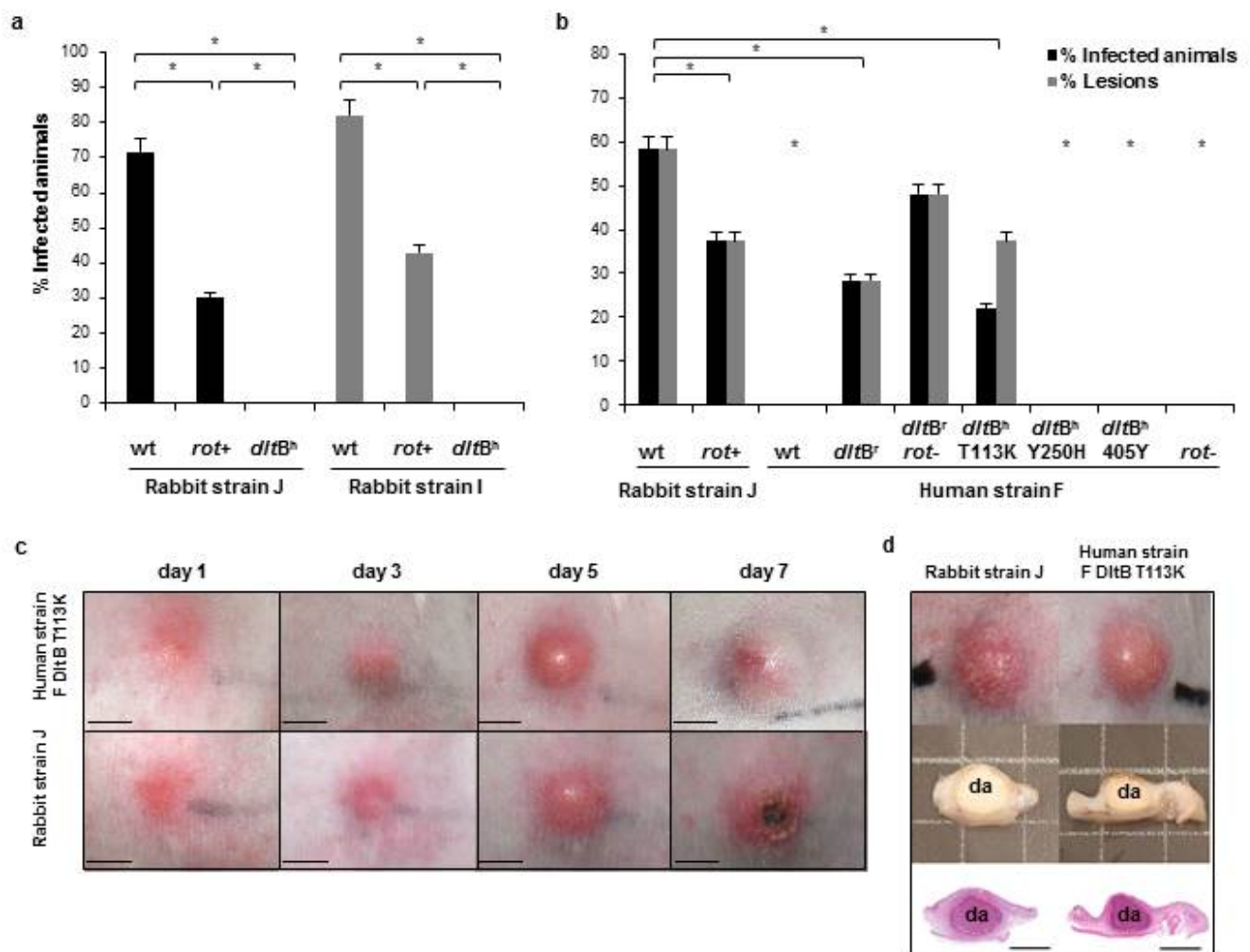
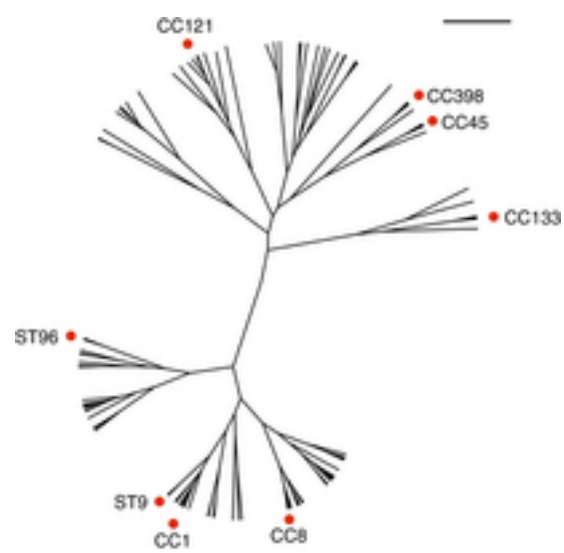


Fig. 2

Fig. 3



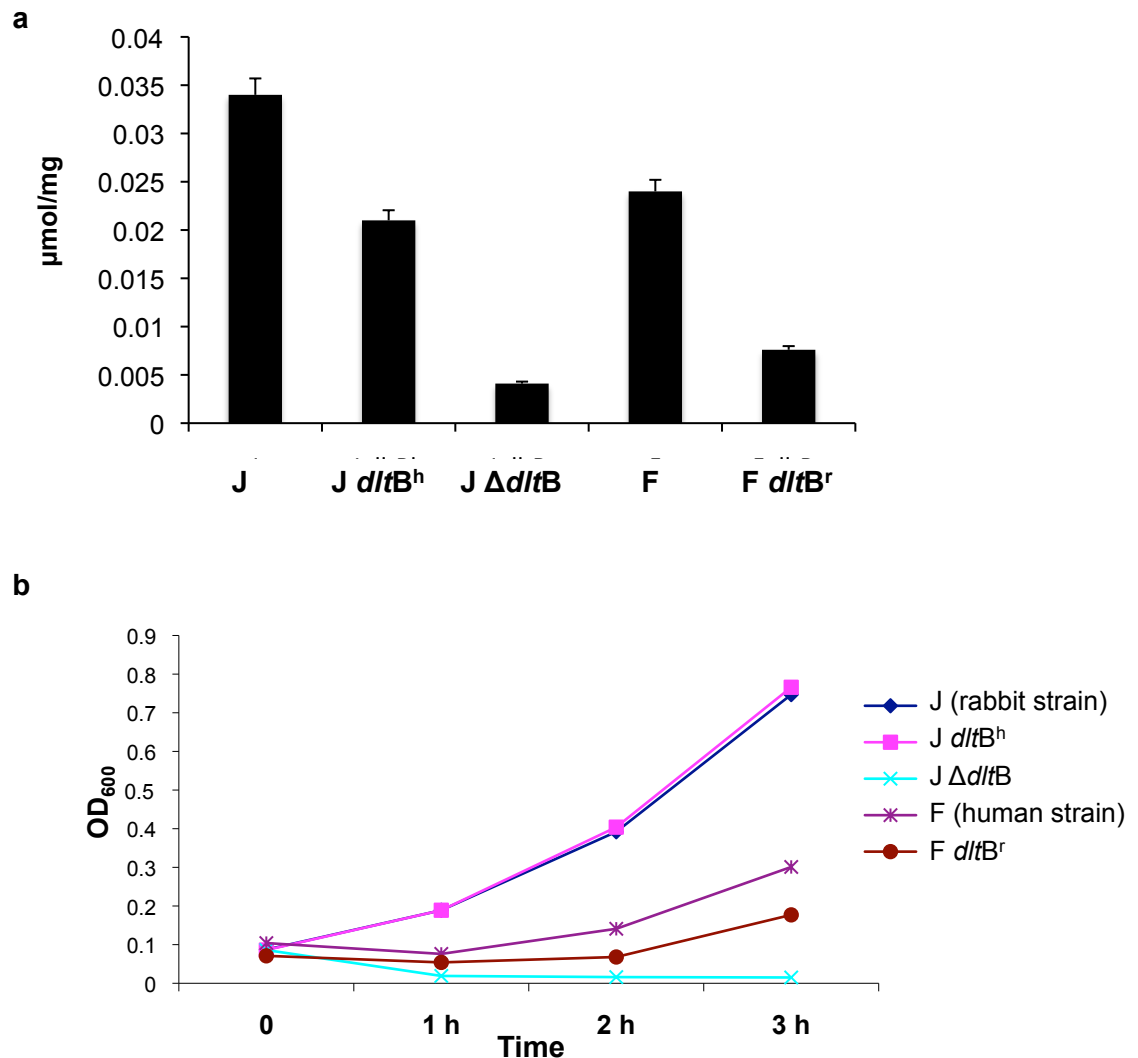


Supplementary Figure 1. Alignment of DltB amino acid sequences from *S. aureus* human, rabbit, ovine and poultry clones, coloured according to relative sequence conservation at each position.

		10	20	30	40	50
Human		MIPYGDFTFF	LIALIALLPV	IILGFLGKRS	YIYNGVVTAF	MIVLIFSSDK
Ovine		MIPYGDFTFF	LIALIALLPV	IILGFLGKRS	YIYNGVVTAF	MIVLIFSSDK
Poultry		MIPYGDFTFF	LIALIALLPV	IILGFLGKRS	YIYNGVVTAF	MIVLIFSSDK
Rabbit ST121		MIPYGDFTFF	LIALIALLPV	IILGFLGKRS	YIYNGVVTAF	MIVLIFSSDK
Consistency		*****	*****	*****	*****	*****
		60	70	80	90	100
Human		HNLFDDQKYL	VQLISFIIYV	VWQVLLIMFY	YHSPKNNNSF	SKFVTVMVLS
Ovine		HNLFDDQKYL	VQLISFIIYV	VWQVLLIMFY	YHSPKNNNSF	SKFVTVMVLS
Poultry		HNLFDDQKYL	VQLISFIIYV	VWQVLLIMFY	YHSPKNNNSF	SKFVTVMVLS
Rabbit ST121		HNLFDDQKYL	VQLISFIIYV	VWQVLLIMFY	YHSPKNNNSF	SKFVTVMVLS
Consistency		*****	*****	*****	*****	*****
		110	120	130	140	150
Human		ILPLALVKVL	QSTWLGGHQI	HFHESKLIEF	VGFLGISYVT	FKSVQLIMEI
Ovine		ILPLALVKVL	QSTWLGGHQI	HFHESKLIEF	VGFLGISYVT	FKSVQLIMEI
Poultry		ILPLALVKVL	QSTWLGGHQI	HFHESKLIEF	VGFLGISYVT	FKSVQLIMEI
Rabbit ST121		ILPLALVKVL	QSKWLGGHQI	HFHESKLIEF	VGFLGISYVT	FKSVQLIMEI
Consistency		*****	*6*****	*****	*****	*****
		160	170	180	190	200
Human		RDGSIKEIKV	WKLIQFISFF	PTISSGPIDR	YKRFVKDDKK	VPTGNEYREL
Ovine		RDGSIKEIKV	WKLIQFISFF	PTISSGPIDR	YKRFVKDDKK	VPTGNEYREL
Poultry		RDGSIKEIKV	WKLIQFISFF	PTISSGPIDR	YKRFVKDDKK	VPTGNEYREL
Rabbit ST121		RDGSIKEIKV	WKLIQFISFF	PTISSGPIDR	YKRFVKDDKK	VPTGNEYREL
Consistency		*****	*****	*****	*****	*****
		210	220	230	240	250
Human		VLKAIHMIML	GFLYKYIVAY	FINTYAIMPL	QLDLHGFEVNL	WLYMYAYSILY
Ovine		VLKAIHMIML	GFLYKYIVAY	FINTYAIMPL	QLDLHGFEVNL	WLYMYAYSILY
Poultry		VLKAIHMIML	GFLYKYIVAY	FINTYAIMPL	QLDLHGFEVNL	WLYMYAYSILY
Rabbit ST121		VLKAIHMIML	GFLYKYIVAY	FINTYAIMPL	QLDLHGFEVNL	WLYMYAYSILH
Consistency		*****	*****	*****	*****	*****7
		260	270	280	290	300
Human		LFFDFAGYSL	FAIAFSYLFG	IKTPPNFDKP	FKAKNIKDFW	NRWHMTLSFW
Ovine		LFFDFAGYSL	FAIAFSYLFG	IKTPPNFDKP	FKAKNIKDFW	NRWHMTLSFW
Poultry		LFFDFAGYSL	FAIAFSYLFG	IKTPPNFDKP	FKAKNIKDFW	NRWHMTLSFW
Rabbit ST121		LFFDFAGYSL	FAIAFSYLFG	IKTPPNFDKP	FKAKNIKDFW	NRWHMTLSFW
Consistency		*****	*****	*****	*****	*****
		310	320	330	340	350
Human		FRDCIYMRSI	FYMSRKLLK	SQFAMSNVAF	LINFFIMGIW	HGIEVYIIVY
Ovine		FRDCIYMRSI	FYMSRKLLK	SQFAMSNVAF	LINFFIMGIW	HGIEVYIIVY
Poultry		FRDCIYMRSI	FYMSRKLLK	SQFAMSNVAF	LINFFIMGIW	HGIEVYIIVY
Rabbit ST121		FRDCIYMRSI	FYMSRKLLK	SQFAMSNVAF	LINFFIMGIW	HGIEVYIIVY
Consistency		*****	*****	*****	*****	*****
		360	370	380	390	400
Human		GLYHAALFIG	YGYERWRKK	HPPRWQNGFT	TALSIVITFH	FVTFGFLIFS
Ovine		GLYHAALFIG	YGYERWRKK	HPPRWQNGFT	TALSIVITFH	FVTFGFLIFS
Poultry		GLYHAALFIG	YGYERWRKK	HPPRWQNGFT	TALSIVITFH	FVTFGFLIFS
Rabbit ST121		GLYHAALFIG	YGYERWRKK	HPPRWQNGFT	TALSIVITFH	FVTFGFLIFS
Consistency		*****	*****	*****	*****	*****
		410	420	430	440	450
Human		GKLI				
Ovine		GKLI				
Poultry		GKLI				
Rabbit ST121		GKLI				
Consistency		*****0				

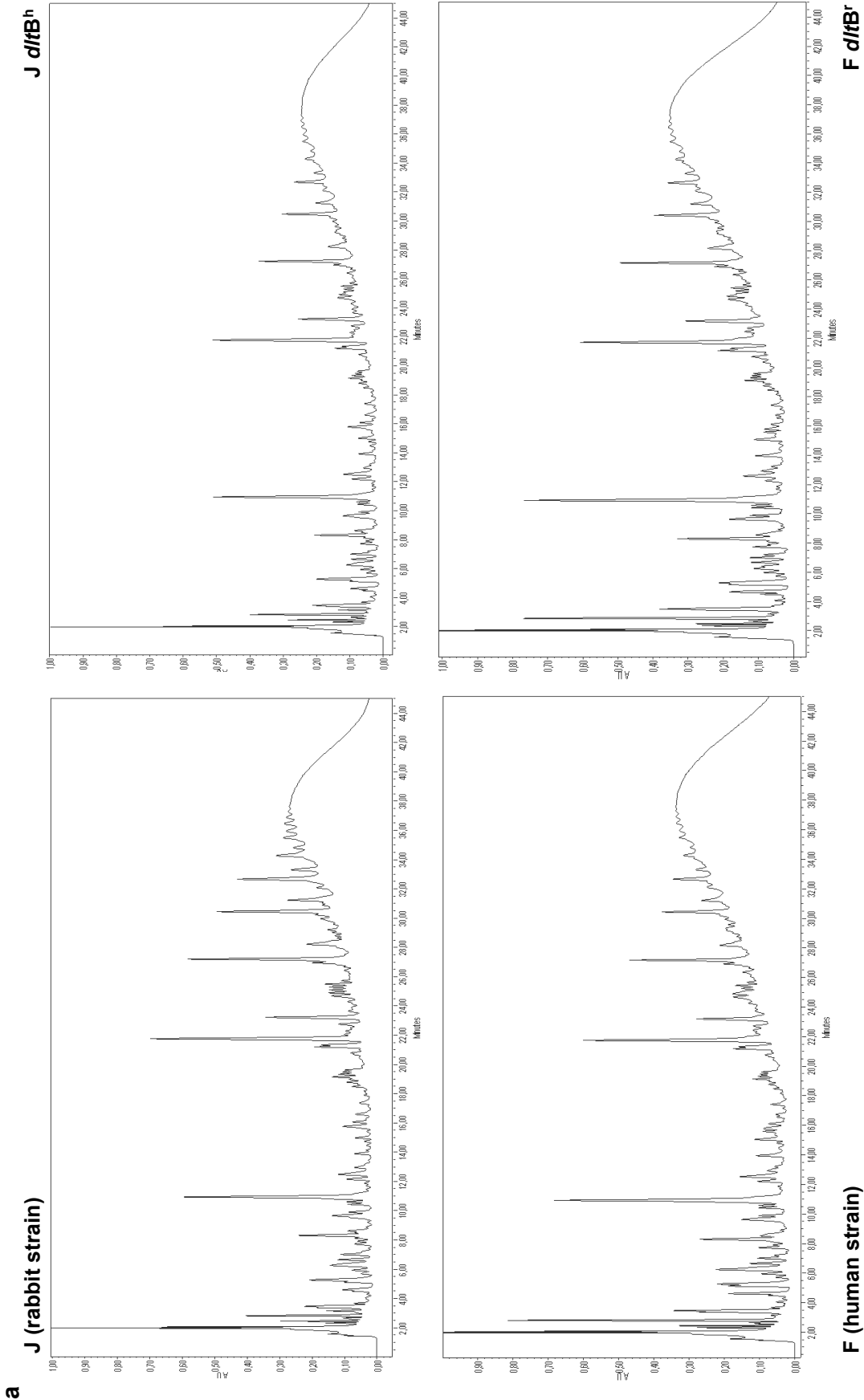
Adapted from alignment generated by PRALINE. The scoring scheme works from 0 for the least conserved alignment position, up to 10 (*) for the most conserved alignment position.

Supplementary Figure 2. Analysis of D-alanylation of wall teichoic acid (a) and growth inhibition by the cationic peptide nisin (b).



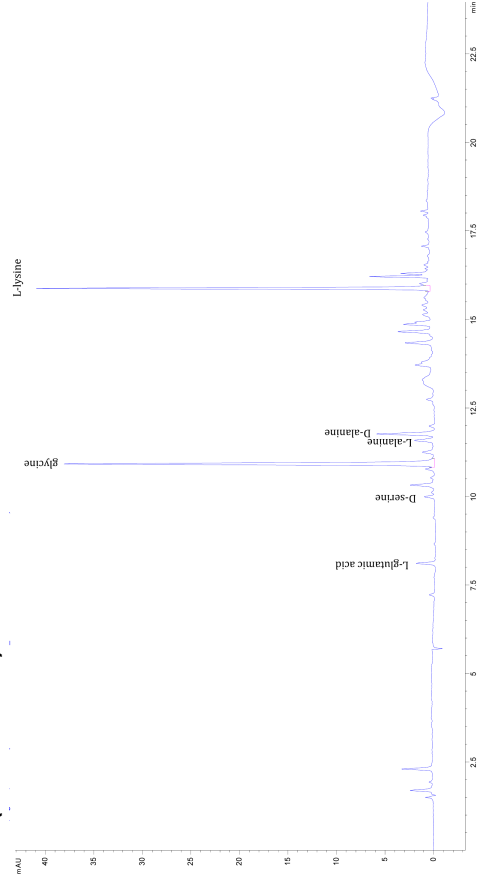
(a) The D-Ala content of the human and rabbit *S. aureus* clones was tested. (b) Shown is the growth in TSB medium of isogenic strains treated with nisin (10 μg/ml). Cell density was monitored (OD₆₀₀). J: wt rabbit strain; J *dltB^h*: Derivative J strain expressing the DltB from the ST121 human clones; J Δ *dltB*: J *dltB* mutant; F: wt human strain; F *dltB^r*: strain F expressing the DltB from the rabbit clones. In both cases, the experiments were done in triplicate. **Statistical analyses are by unpaired t tests.** All error bars show s.e.m.

Supplementary Figure 3. Analysis of the peptidoglycan structure and composition .

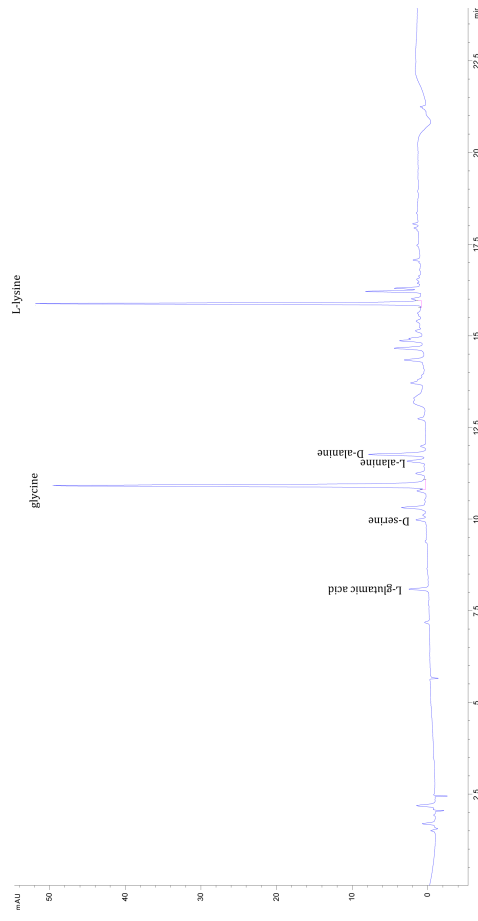


b

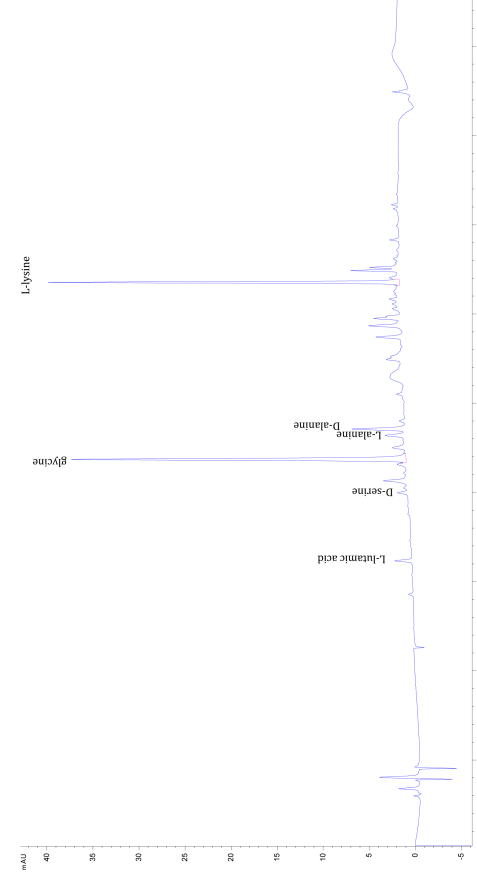
J (rabbit strain)



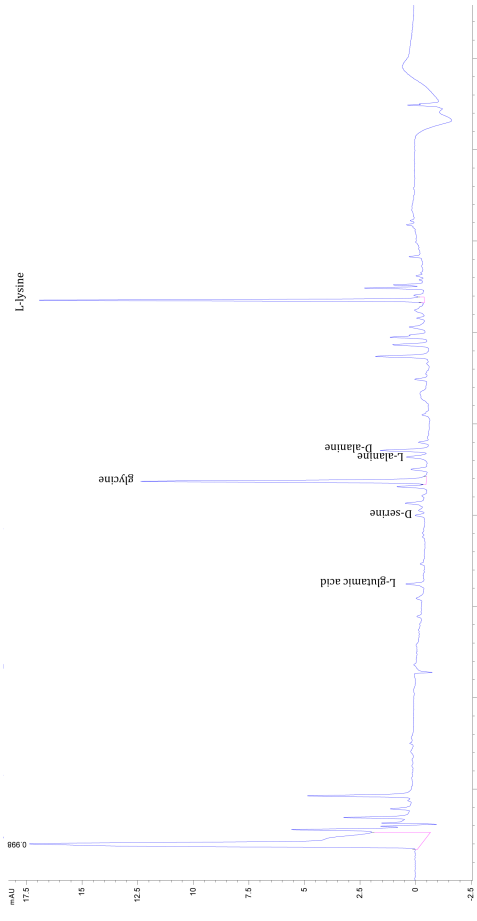
J d/tB^h



F (human strain)

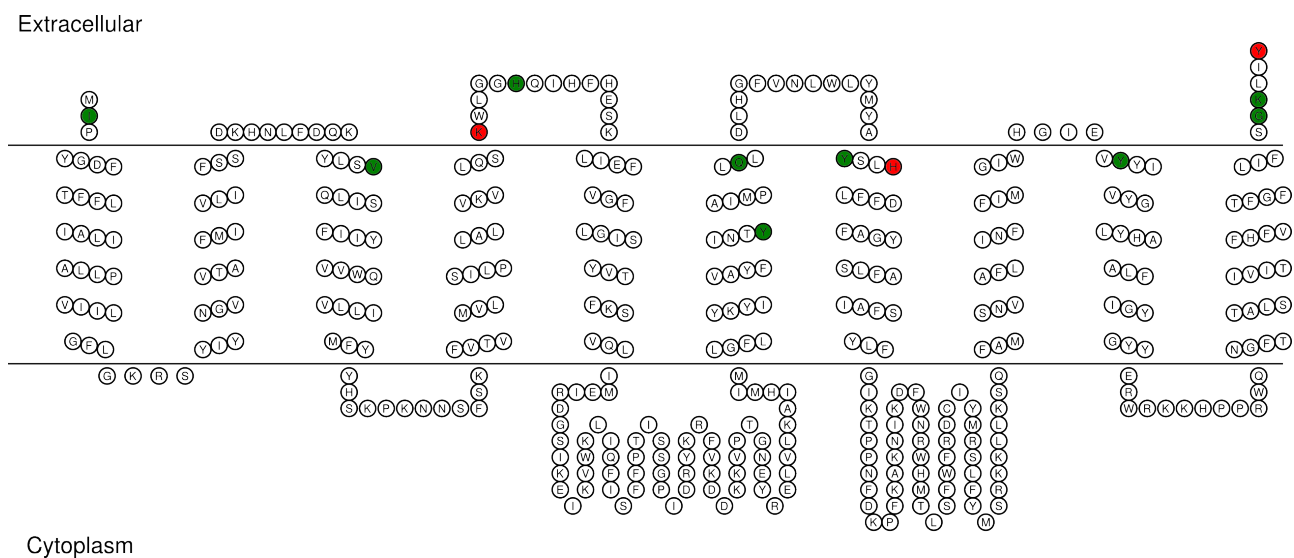


F d/tB^r



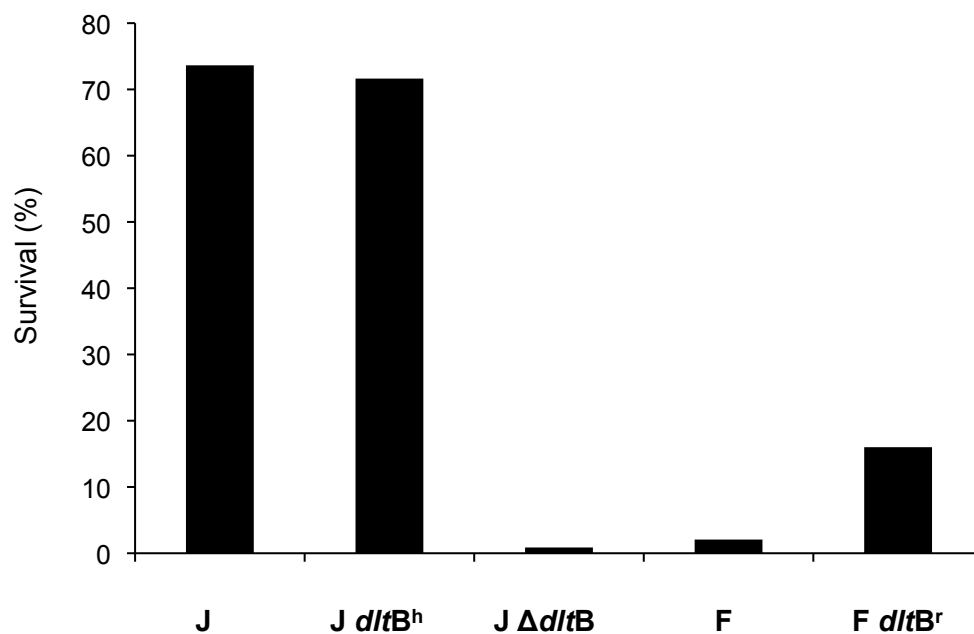
(a) Muropeptide analysis by HPLC. The cell wall from bacteria growing in exponential phase was isolated, then digested with a muramidase, and analyzed via HPLC. (b) The amino acid composition of the purified peptidoglycan was analyzed by HPLC. In both panels, a representative experiment is shown.

Supplementary Figure 4. Predicted membrane topology of the DltB protein.



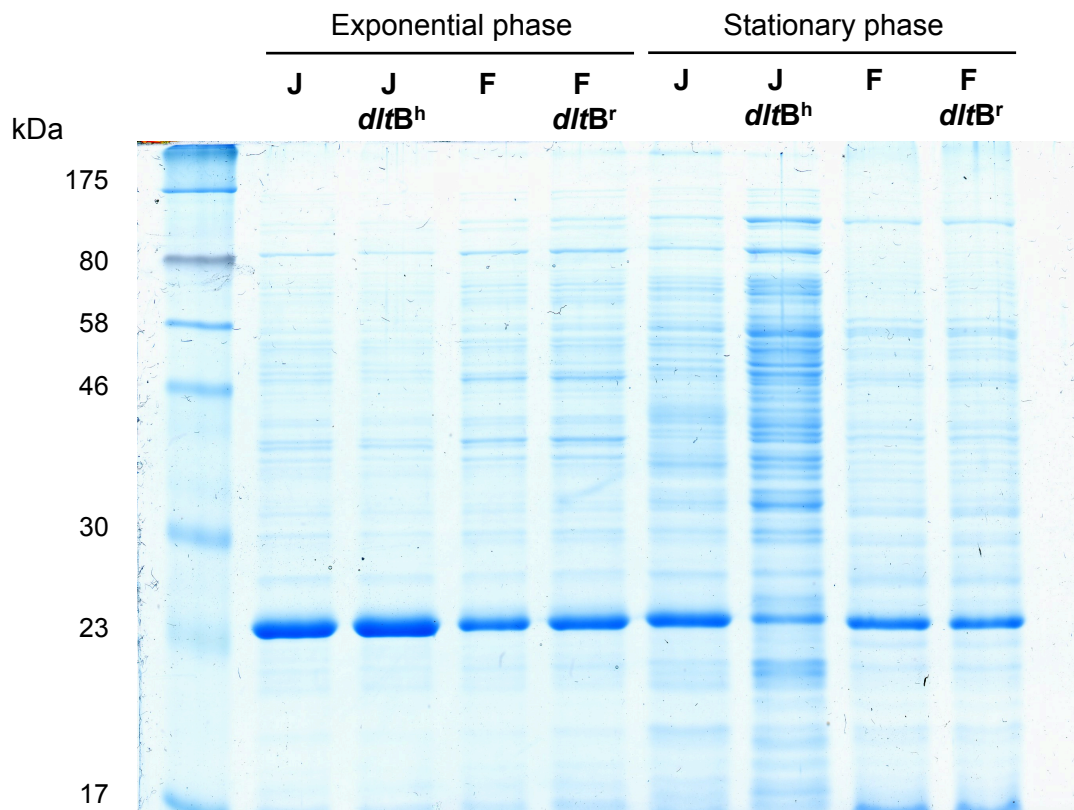
DltB topology was predicted using the TMHMM method. Coloured in red are amino acid residues that varied in rabbit ST121 strains, and green indicates amino acid residues that were variant in the other rabbit clones.

Supplementary Figure 5. Survival in rabbit blood.



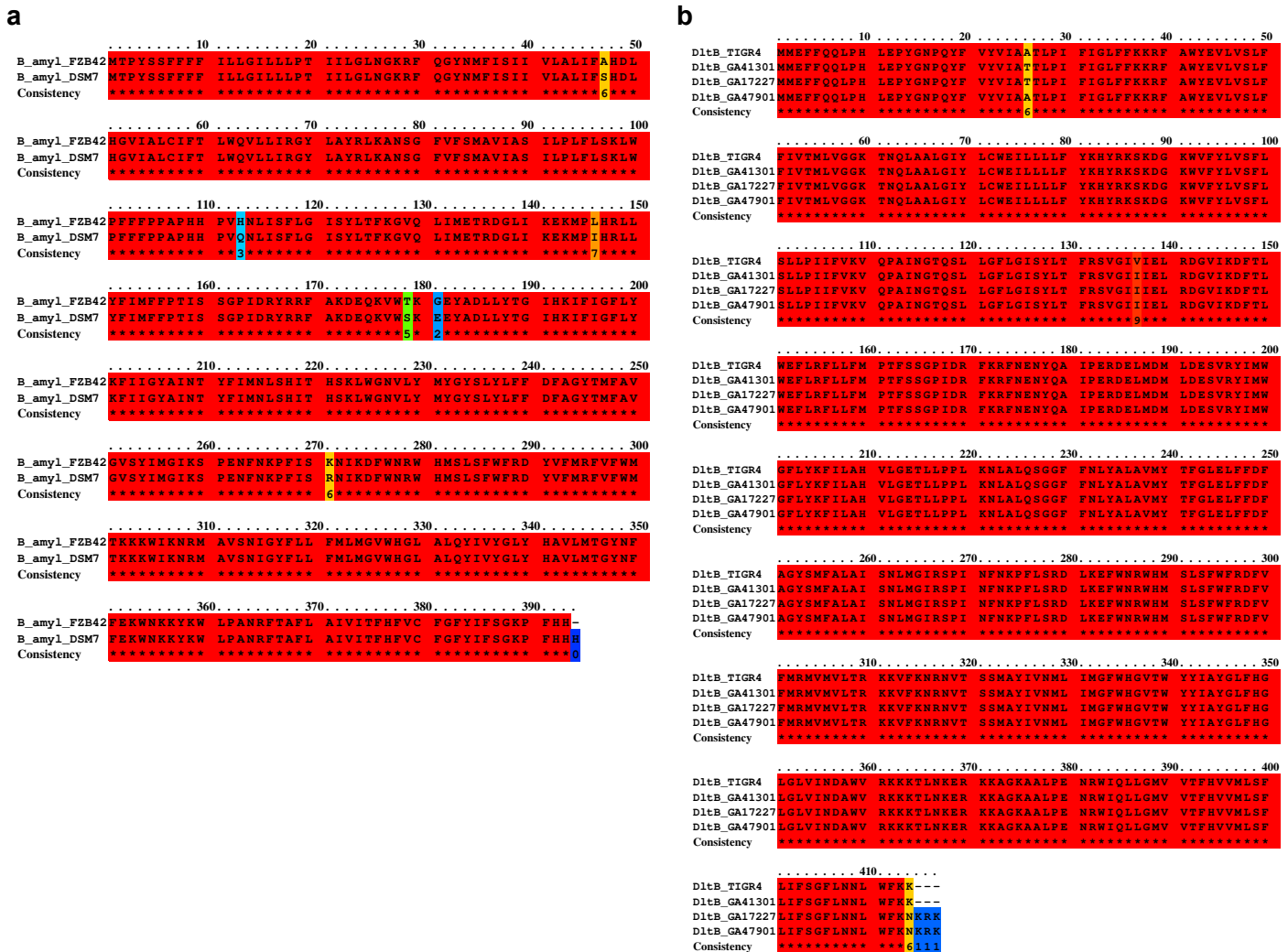
S. aureus strains were grown to mid-exponential growth phase, washed, and resuspended in sterile PBS. 4×10^4 CFU of *S. aureus* in 100 μ l PBS were pipetted slowly into 3 ml heparinized rabbit blood, mixed gently for 30 s and incubated at 37 °C for 2 h. To determine survival rates, 100 μ l of heparinized blood was plated on TSA for *S. aureus* detection. Data shown represent mean \pm s.e.m. of three separate experiments. **Statistical analyses are by unpaired t tests.**

Supplementary Figure 6. SDS PAGE analysis of cell-wall-associated protein profiles



S. aureus strains were grown to mid-exponential or stationary growth phase, washed, and resuspended in lysis buffer (50mM Tris-HCl pH7.5, 20mM MgCl₂, supplemented with 30% raffinose) with lysostaphin. Protoplasts were sedimented by centrifugation at 6,000 x *g*, and the supernatant fraction, which contained the wall-associated proteins, was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Supplementary Figure 7. Alignment of DltB amino acid sequences from (a) *Bacillus amyloliquefaciens*, and (b) *Streptococcus pneumoniae* strains, coloured according to relative sequence conservation at each position.



Adapted from alignment generated by PRALINE. The scoring scheme works from 0 for the least conserved alignment position, up to 10 (*) for the most conserved alignment position. (a) B_aml1_FZB42: *Bacillus amyloliquefaciens* strain FZB42 (plant-associated bacterium). Accession number: YP_001423132. B_aml1_DSM7: *Bacillus amyloliquefaciens* strain DSM7 (soil-adapted). Accession number: YP_003922279. (b) DltB_TIGR4: *S. pneumoniae* strain TIGR4. Accession number: ZP_01408978. DltB_GA41301: *S. pneumoniae* strain GA41301. Accession number: ZP_12336902. DltB_GA17227: *S. pneumoniae* strain GA17227. Accession number: ZP_12797610. DltB_GA47901: *S. pneumoniae* strain GA47901. Accession number: ZP_12344230.